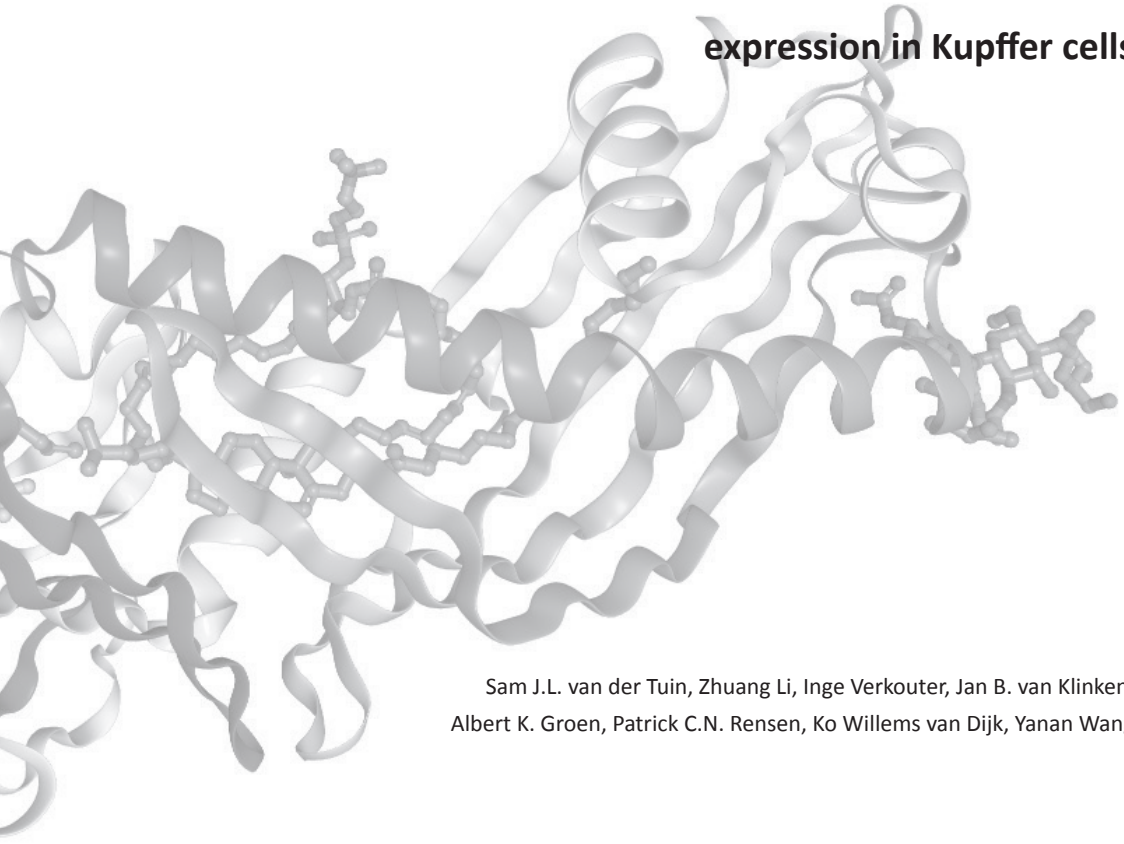
Novel mechanistic insight in cholesteryl ester transfer protein production and pharmacological inhibition

Tuin, S.J.L. van der

Chapter 4

Lipopolysaccharide increases HDL-cholesterol by reducing cholesteryl ester transfer protein expression in Kupffer cells



Sam J.L. van der Tuin, Zhuang Li, Inge Verkouter, Jan B. van Klinken, Albert K. Groen, Patrick C.N. Rensen, Ko Willems van Dijk, Yanan Wang

ABSTRACT

Kupffer cells, the resident hepatic macrophages, play an important role in immune surveillance of the liver. Since we have previously shown that Kupffer cells are the main source of plasma cholesteryl ester transfer protein (CETP), Kupffer cells may function as a link between the immune status of the liver and plasma levels of pro- and anti-inflammatory plasma lipoproteins. To address this hypothesis, we investigated the effect of intraperitoneal lipopolysaccharide (LPS) injection on hepatic macrophage activation, CETP expression, and plasma lipid and lipoprotein levels. Four and 8 hours after injection of LPS, a marked decrease in the expression of the resident Kupffer cell markers C-type lectin domain family 4, member f (*Clec4f*) and V-set and immunoglobulin domain containing 4 (*Vsig4*) was apparent, which coincided with a decrease in the expression of CETP and an increase in the expression of the pro-inflammatory genes tumor necrosis factor α (*Tnfα*) and monocyte chemotactic protein-1 (*Mcp-1*). Simultaneously, the ratio of plasma high density lipoprotein cholesterol (HDL-C) over non-HDL-C transiently peaked. Eight hours after LPS injection, expression of the infiltrating monocyte marker *Ly6c* was increased, concomitant with an increase in the number of $Ly6c^+$ cells in the liver. Collectively, these data indicate that upon LPS injection, Kupffer cells are rapidly activated, lose expression of resident Kupffer cell markers and CETP expression increases the HDL-C/nonHDL-C ratio. These data show that hepatic macrophages link the response to the inflammatory stimulus LPS with an increase in the level of HDL-C via down regulation of CETP expression.

INTRODUCTION

Kupffer cells are the resident tissue macrophages of the liver and play an important role in the early detection and response to harmful agents such as intestinal-derived bacterial lipopolysaccharide (LPS) in the portal vein. Kupffer cells have been implicated in the pathology of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis that are strongly associated with obesity. We have recently shown that plasma cholesteryl ester transfer protein (CETP) is predominantly derived from Kupffer cells, and that plasma CETP concentration predicts hepatic macrophage content in humans.[1] However, only 39% of $CD68^+$ Kupffer cells co-express CETP in humans, and 57% of Kupffer cells in the liver co-express CETP in human CETP transgenic mice.[1] To evaluate which hepatic macrophage population(s) express(es) CETP, macrophages were eliminated from the livers of APOE*3-Leiden.CETP transgenic mice using liposomal clodronate and their repopulation was followed in time. From these data, we concluded that CETP is expressed by $F4/80^+Ly6C^-$ $Clec4f^+Vsig4^+$ macrophages in the liver representing resident mature Kupffer cells, but not by $F4/80^+Ly6C^+Clec4f^-Vsig4^-$ macrophages representing infiltrated immature macrophages (**Chapter 3**).

CETP mediates the exchange of cholesteryl ester (CE) for triglyceride (TG) between high density lipoprotein (HDL) and (very) low density lipoprotein [(V)LDL]. As such, CETP activity decreases HDL-C and increases (V)LDL-C, which results in a lipoprotein profile that is associated with an increased risk for cardiovascular disease (CVD). This has led to the development of pharmacological CETP inhibitors as alternative or supplemental treatment to statins for the improvement of dyslipidemia characterized by high levels of (V)LDL and low HDL. Despite clearly favourable effects on the lipoprotein profile, pharmacological CETP inhibitors, such as torcetrapib,[2] dalcetrapib[3] and evacetrapib[4], failed to show beneficial effects on CVD outcomes. These results illustrate that the role and underlying mechanism of CETP activity in CVD pathology is more complex than initially anticipated.

Besides playing a role in lipid and lipoprotein metabolism, CETP belongs to the family of lipid transfer/LPS-binding proteins, which might also function in the innate immune response.[5] Lipopolysaccharide (LPS), a constituent of Gram-negative bacteria, is a potent endotoxin that induces a strong cytokine-mediated systemic inflammatory response in the host.[6] LPS binds the TLR4/CD14/MD2 complex, which induces the release of proinflammatory cytokines and inflammation, and is an underlying cause of acute sepsis but also chronic inflammatory disorders.[7] Previous studies have suggested that CETP is important as a host defense mechanism against LPS-induced systemic inflammation. Administration of LPS to CETP transgenic mice resulted in a rapid and marked decrease in plasma CETP concentration and hepatic CETP expression, accompanied with an increase in HDL-C level.[8] Taking into account the well-documented anti-inflammatory properties of HDL,[9, 10] reducing CETP

concentration represents an adaptive response to preserve or increase HDL levels, thereby decreasing LPS toxicity and increasing host survival. However, the mechanism(s) by which LPS reduces plasma CETP concentration and hepatic CETP expression is still rather obscure.

In the current study, we aimed to study the role of hepatic macrophages in the mechanism by which LPS reduces plasma CETP concentration and hepatic CETP expression and increases HDL-C. To address this question, APOE*3-Leiden.CETP transgenic mice were injected with LPS and the hepatic macrophage activation status and CETP expression, as well as the plasma lipid and lipoprotein profiles, were determined in time.

MATERIALS AND METHODS

Animals

Female APOE*3-Leiden.CETP transgenic mice[11] were housed under standard conditions with a 12 h light-dark cycle and had free access to food and water during the experiment. At the age of 10-15 weeks, mice were fed a semi-synthetic cholesterol-rich diet, containing 15% (w/w) cacao butter and 0.1% cholesterol (Western-type diet; AB-Diets) for a run-in period of 6 weeks. After randomization according to plasma levels of triglycerides, total cholesterol, HDL-C and body weight, mice received intraperitoneal injections of LPS (25 µg per mouse) in phosphate-buffered saline and were terminated 4 hours, 8 hours and 48 hours after the injection. Control mice received intraperitoneal injections of vehicle (LPS free phosphate-buffered saline). All animals were sacrificed by CO_2 inhalation. The Institutional Ethics Committee for Animal Procedures from the Leiden University Medical Centre, Leiden, The Netherlands, approved the protocol.

Blood sampling, plasma lipid and lipoprotein profiles

Blood was obtained via tail vein bleeding into heparin-coated capillary tubes. The tubes were placed on ice and centrifuged, and obtained plasma was snap-frozen in liquid nitrogen and stored at -80°C until further measurements. Plasma was assayed for triglycerides and cholesterol using the commercially available enzymatic kits 11488872 and 236691 (Roche Molecular Biochemicals), respectively. To measure plasma HDL-C, apoB-containing lipoproteins were precipitated from plasma with 20% polyethylene glycol 6000 (Sigma Aldrich) in 200 mM glycine buffer (pH 10) and HDL-C was measured in the supernatant. Plasma nonHDL-C was calculated by subtracting HDL-C from plasma total cholesterol.

Plasma CETP concentration

Plasma CETP concentration was measured using the DAIICHI CETP ELISA kit according to manufacturer's instructions (Daiichi).

Hepatic gene expression

Liver pieces were isolated and total RNA was extracted using the Nucleospin RNAII kit (Macherey-Nagel) according to manufacturer's protocol. RNA concentration was determined by Nanodrop technology (Thermo Scientific). Total RNA was reverse-transcribed with iScript cDNA synthesis kit (Bio-Rad) and qPCR was performed using a CFX96™ (Bio-Rad). Gene expression was normalized to Beta-2 microglobulin, hypoxanthine ribosyltransferase and Beta-actin. Relative expression was calculated and normalized to control group using Bio-Rad CFX Manager™ software 3.0 (Bio-Rad). Primer sequences can be found in the Supplemental Table 1.

Table S1: Primer sequences used for qPCR

Gene	Forward primer	Reverse Primer
B-actin	AACCGTGAAAAGATGACCCAGAT	CACAGCCTGGATGGCTACGTA
ß-2m	TGACCGGCTTGTATGCTATC	CAGTGTGAGCCAGGATATAG
CETP	CATGTCTCGGCTCGAGGTAG	TTCTGCTACAAGCCCCATCC
Clec4f	ACTGAAGTACCAAATGGACAATGTTAGT	GTCAGCATTCACATCCTCCAGA
F4/80	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
Hprt	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
Ly6C	CTGCAACCTTGTCTGAGAGGA	GTCCCTGAGCTCTTTCTGCAC
Vsig4	TCACCTATGGCCACCCCACC	AGGCGGCCTCTGTACTTTGCCT

ß-actin, Beta-actin; ß-2m, ß-2 microglobulin; *CETP*, cholesteryl ester transfer protein; *Clec4f*, C-type lectin domain family 4, member f; *Hprt*, hypoxanthine ribosyltransferase; *Vsig4*, V-set and immunoglobulin domain containing 4.

Liver histology

Paraffin-embedded sections of mouse liver (5 µm) were stained for F4/80, Clec4f (MAB2784; 1/1000, R&D Systems), Vsig4 (AF4674; 1:1000, R&D Systems), Ly6C (ab15627; 1/400, Abcam) and for human CETP (ab51771; 1/1000, Abcam) as described previously.[1]

Statistical analysis

Significance of differences between the groups was calculated non-parametrically using a Mann-Whitney U-test for independent samples. All groups were compared to the control group. Values are presented as mean±SD. P-values of less than 0.05 were considered statistically significant for single comparison. Bonferroni's method was used to determine significance in case of multiple comparisons. SPSS 18.0 for Windows was used for statistical analysis.

RESULTS

LPS reduces hepatic expression of CETP, in parallel with reduced expression of mature Kupffer cell markers and increased expression of pro-inflammatory markers

To investigate the time-dependent effects of LPS administration on the gene expression profile of hepatic macrophage markers, APOE*3-Leiden.CETP mice were injected intraperitoneally with LPS or vehicle (control group). APOE*3-Leiden.CETP mice were sacrificed before and 4, 8 and 48 hours after LPS injection, and liver samples were collected. LPS rapidly and markedly decreased hepatic mRNA expression of CETP (up to -75% at 8 hours after injection; P<0.001, Fig. 1A). At 48 hours after LPS injection CETP expression was still reduced. Next, the hepatic gene expression of C-type lectin domain family 4, member f (*Clec4f*) and V-set and immunoglobulin domain containing 4 (*Vsig4*) were measured, both of which are markers of tissue resident mature macrophages.[12-14] LPS markedly decreased the expression of mature macrophage markers *Clec4f* (up to -75% at 8 hours after injection; P<0.001, Fig. 1B) and *Vsig4* (up to -83% at 8 hours after injection; P<0.001, Fig. 1C) in liver. In contrast, LPS increased hepatic mRNA expression of *Ly6c*, a marker of infiltrating monocytes/macrophages (up to +48%; P<0.001 at 8 hours after injection, Fig. 1D). In addition, a massive upregulation of mRNA expression of the cytokine tumor necrosis factor α (*Tnfα*, up to 21-fold higher at 4 hours after injection; P<0.001, Fig. 1E) and the chemokine monocyte chemotactic protein-1 (*Mcp-1*, up to 28-fold higher at 4 hours after injection; P<0.001, Fig. 1F) were observed in liver after LPS injection, indicating LPS-induced Kupffer cell activation. Notably, the hepatic gene expression of Tnfα and Mcp-1 returned to baseline levels at 48 hours after LPS injection, indicating that the induction of these proinflammatory genes represents an acute, yet transient response.

Correlation analyses showed that hepatic *CETP* expression strongly positively correlated with the expression of *Clec4f* (r=0.735; P<0.001) and *Vsig4* (r=0.904; P<0.001), both of which are markers of resident mature Kupffer cells, whereas *CETP* inversely correlated with the proinflammatory markers Tnfα (r=-0.614; P<0.001) and *Mcp-1* (r=-0.502; P<0.01). In contrast, hepatic *CETP* expression was not associated with *Ly6c* (r=0.009), a marker of infiltrating monocytes/macrophages.

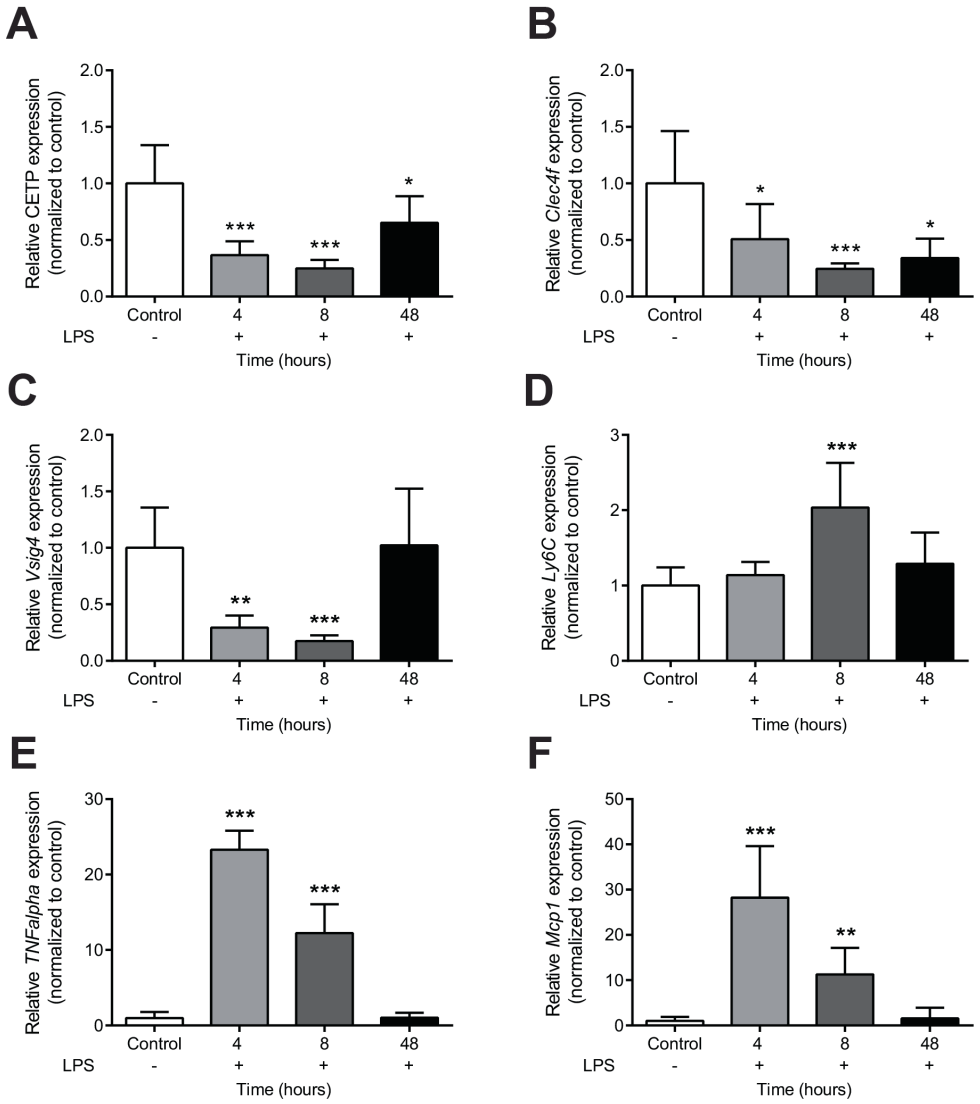


Figure 1: LPS reduces hepatic expression of CETP, in parallel with reduced expression of mature Kupffer cell markers and increased expression of pro-inflammatory markers.
Female APOE*3-Leiden.CETP mice fed a Western-type diet were intraperitoneally injected with 25 µg LPS or vehicle (control), after which mice were sacrificed at the indicated time points. Livers were assayed for mRNA of *CETP* (A), *Clec4f* (B), *Vsig4* (C), *Ly6c* (D), *Tnfα* (E) and *Mcp-1* (F).
Data are presented as mean±SD (n=7-8); *P<0.05, **P<0.01, ***P<0.001 as compared to the control group.

LPS reduces plasma CETP, triglycerides, total cholesterol and non-HDL cholesterol, and transiently increases plasma HDL-cholesterol and HDL-C/nonHDL-C ratio

Plasma CETP, lipid and lipoprotein concentrations were assayed at baseline and 8 hours, 1 day, 2 days, 3 days and 7 days after LPS injection in APOE*3-Leiden.CETP mice. Compared to the control group, LPS rapidly reduced plasma CETP concentration already at 8 hours after injection (-51%; P<0.01, Fig. 2). The LPS-induced reduction in plasma CETP concentration persisted until 7 days after injection (-61%; P<0.01, Fig. 2). Plasma TG level was reduced 1 day after LPS injection, which returned to the baseline 7 days after LPS injection (Fig. 3A). LPS rapidly decreased plasma total cholesterol (C) concentration (Fig. 3B), accompanied with decreased nonHDL-C (Fig. 3C). In contrast, LPS transiently increased HDL-C concentration 8 hours after injection (+81%; P=0.12, Fig. 3D). To investigate the effect of LPS on the distribution of cholesterol over plasma lipoproteins, the ratio between HDL-C and nonHDL-C was calculated (Fig. 3E). The ratio revealed that LPS induced a rapid and transient shift of cholesterol from nonHDL lipoproteins to HDL fraction, while the ratio was normalized to baseline after 1 day of LPS injection.

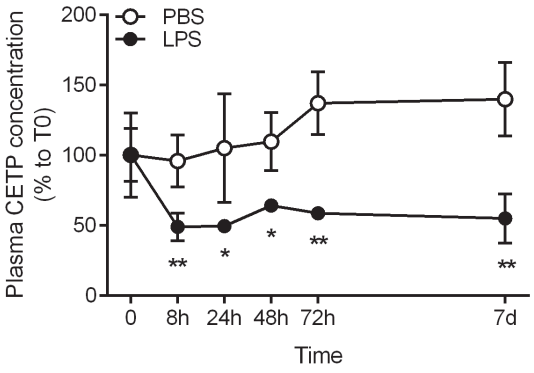


Figure 2: LPS rapidly reduces plasma CETP concentration

Female APOE*3-Leiden.CETP mice fed a Western-type diet were intraperitoneally injected with 25 µg LPS or vehicle (control), after which blood samples were collected at the indicated time points. Plasma was assayed for CETP concentration and data are expressed relative to t=0.

Data are presented as mean±SD (n=7); *P<0.05, **P<0.01 as compared to the control group.

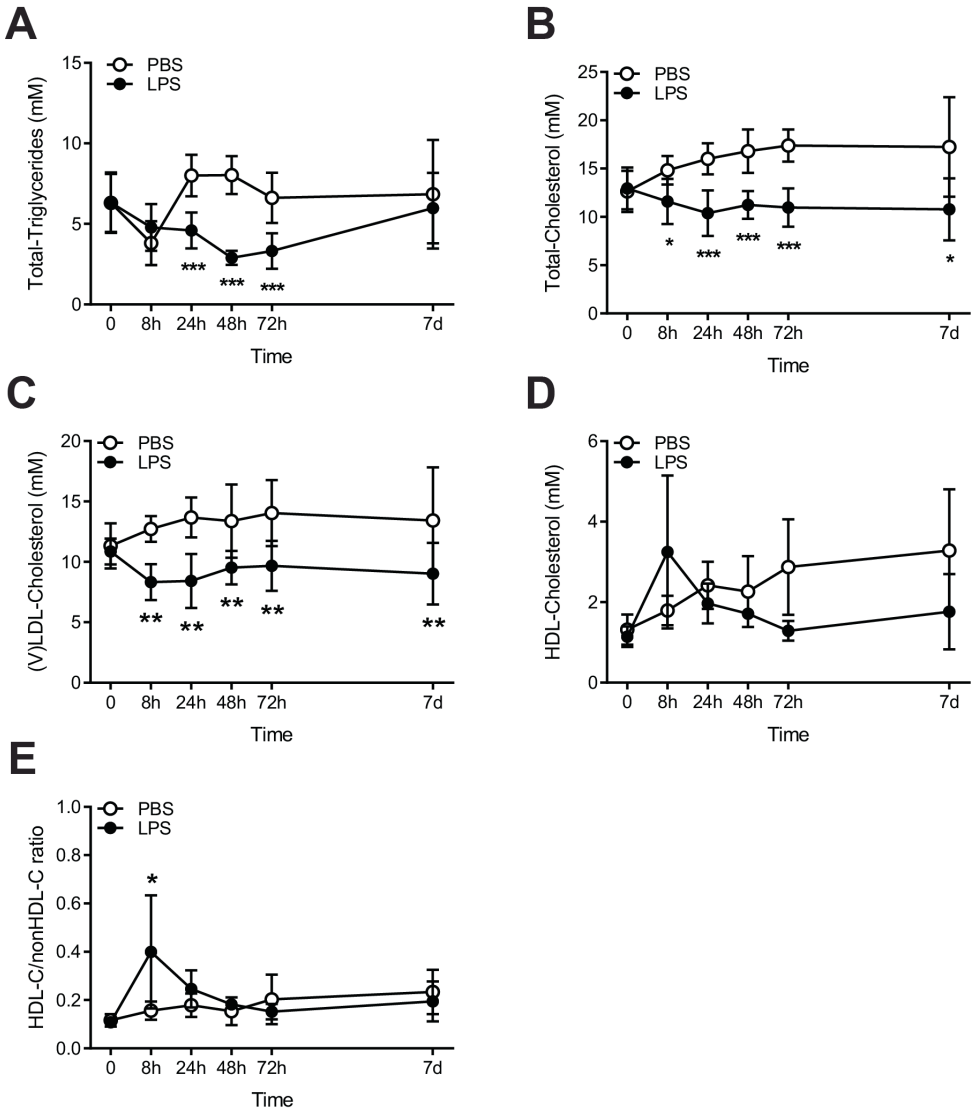


Figure 3: LPS reduces plasma triglycerides, total cholesterol and non-HDL cholesterol, and transiently increases plasma HDL-cholesterol and HDL-C/nonHDL-C ratio

Female APOE*3-Leiden.CETP mice fed a Western-type diet were intraperitoneally injected with 25 µg LPS or vehicle (control), after which blood samples were collected at the indicated time points. Plasma was assayed for triglyceride (A), total cholesterol (B), nonHDL-cholesterol (C) and HDL-cholesterol (D), and the ratio between HDL-C and nonHDL-C (E) was calculated.

Data are presented as mean±SD (n=7); *P<0.05, **P<0.01, ***P<0.001 as compared to the control group.

LPS does not affect hepatic macrophage content, but significantly decreases mature Kupffer cell content and increases immature macrophage recruitment to the liver

Next, immunohistochemistry (IHC) was performed on liver sections and the numbers of F4/80$^+$, CETP$^+$, Clec4f$^+$ and Ly6c$^+$ cells were quantified. After LPS administration, hepatic macrophage content was not affected as shown by the number of F4/80$^+$ cells, despite a slight non-significant increase at 4 and 8 hours after injection (Fig. 4A). LPS tended to decrease the number of CETP$^+$ cells 48 hours after injection (-20%; P=0.06, Fig. 4B), which coincides with a significantly decreased number of Clec4f$^+$ resident mature Kupffer cells 48 hours after injection (Fig. 4C). At 4 and 8 hours after LPS injection the numbers of CETP$^+$ cells and the numbers of Clec4f$^+$ cells were not affected, presumably due to relatively slow protein turnover as compared to mRNA turnover. In line with the observation that LPS markedly increased the gene expression of the infiltrating monocyte marker Ly6c, LPS significantly increased Ly6c$^+$ infiltrating monocyte recruitment to the liver (Fig. 4D) 4 and 8 hours after LPS injection.

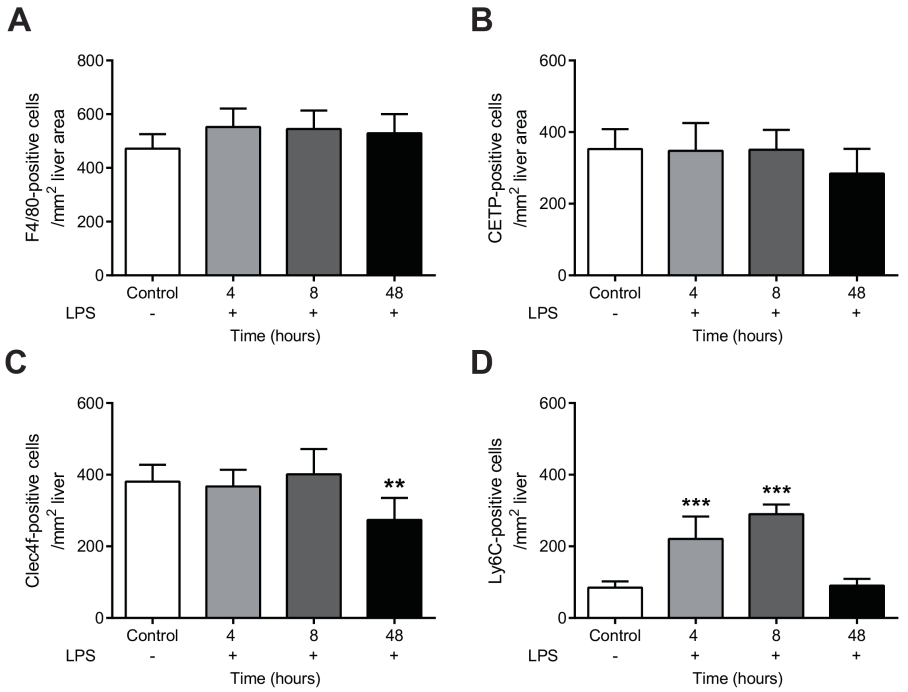


Figure 4: LPS does not influence hepatic macrophage content, but largely increases immature macrophage recruitment to the liver and significantly decreases mature Kupffer cell content

Female APOE*3-Leiden.CETP mice fed a Western-type diet were intraperitoneally injected with 25 µg LPS or vehicle (control), after which mice were sacrificed at the indicated time points. Livers were assayed for F4/80$^+$ cells (A), CETP$^+$ cells (B), Clec4f$^+$ cells (C) and Ly6c$^+$ cells (D).

Data are presented as mean±SD (n=7-8); ***P<0.001 as compared to the control group.

DISCUSSION

In the present study, we investigated the role of hepatic macrophages in the mechanism by which LPS reduces plasma CETP concentration and hepatic *CETP* expression. Our data show that LPS strongly reduced hepatic expression of CETP in parallel with strongly reduced expression of mature Kupffer cell markers, while LPS increased expression of activated Kupffer cell markers. The reduction in *CETP* expression caused a shift of plasma cholesterol from non-HDL lipoproteins to the HDL fraction. These data suggests that hepatic macrophages function as a link between the acute inflammatory response of the liver to LPS and the plasma lipoprotein composition via regulation of *CETP* expression.

We have previously demonstrated that the liver is the main source of plasma CETP, and that Kupffer cells are responsible for hepatic expression of *CETP* in humans and CETP transgenic mice[1]. In the current study, we showed that LPS reduced hepatic CETP expression without affecting total hepatic macrophage content as shown by the number of $F4/80^+$ cells, indicating that LPS reduced CETP expression in macrophages per se. This finding is concordance with in vitro data showing that LPS reduced CETP mRNA expression in bone marrow-derived macrophages from CETP transgenic mice and in human monocyte-derived macrophages.[15] In addition to LPS, inflammatory stimuli such as TNFα and INFγ[15] also reduced CETP expression in macrophages. Moreover, Ye et al[16] showed that inflammation induced by acute myocardial infarction decreased hepatic CETP expression as well as the hepatic expression of the CETP/CD68 ratio, indicativ e for reduced macrophage CETP expression in liver. Although the specific stimuli responsible for hepatic macrophages to express CETP have not yet been fully delineated, based on previous studies, we postulate that the presence of both liver-X-receptor α (*LXRα*)[17] and farnesoid-X-receptor (*FXR*) signaling[18] in the liver may be crucial for this capability.

More importantly, we recently showed that CETP is exclusively expressed by $F4/80^+Ly6C^-Clec4f^+Vsig4^+$ macrophages in liver, which represent resident mature Kupffer cells, rather than $F4/80^+Ly6C^+Clec4f^-Vsig4^-$ macrophages in liver representing infiltrating monocytes/immature macrophages (**Chapter 3**). In the current study, we obtained data supporting this notion. We clearly observed that LPS administration quickly reduced the expression of Clec4f and Vsig4, and increased the expression of Ly6c in liver without affecting F4/80 expression, accompanied by reduced hepatic expression of CETP. Taken together, these data indicate that LPS rapidly activates $F4/80^+Ly6C^-Clec4f^+Vsig4^+$ resident Kupffer cells to lose expression of Clec4f and Vsig4 macrophages, thereby abolishing hepatic CETP expression and resulting in a reduced plasma CETP concentration.

To investigate the possible mechanisms underlying the coexpression of CETP and VSIG4 in humans, we inspected the transcription factor ChIP-seq datasets generated by the ENCODE Consortium.[19] Specifically, we selected transcription factor binding sites within a

distance of +/-10 kbp of the transcription start sites (TSS) of both genes, and searched for overlapping transcription factors in monocyte/macrophage-like cell lines. We found that CCAAT/enhancer-binding protein beta (C/EBPB) binds close to the TSS of both genes in the K562 cell line with maximum strength of the ChIP-seq signal. The C/EBPB is a member of the C/EBP family of basic region-leucine zipper proteins and is known to play an important role in macrophage functioning, including macrophage polarization, maturation and antibacterial activity of macrophages.[20, 21] Collectively, these data suggest that C/EBPB is a possible candidate for regulation of CETP expression in Kupffer cells, which is of interest and needs be investigated by future studies.

It is known that CETP belongs to the LPS binding protein (LBP) family, which includes phospholipid transfer protein, bactericidal permeability increasing protein and LBP itself. However, CETP has a low binding affinity to LPS (Kd>25 mM), as compared to LBP (Kd=0.8 nM) and BPI (Kd=0.5 nM).[22] Therefore, CETP likely only plays a role in LPS binding in the acute phase of LPS exposure, when LPS concentration is high. This may explain the observation that CETP expression markedly improves the mouse survival rate after injection of a lethal dose of LPS.[23]On the other hand, CETP plays a pivotal role in lipid and lipoprotein metabolism in humans. After secretion into the circulation, CETP protein binds mainly to HDL, and promotes bidirectional transfer of CE, TG, and to lesser extent phospholipid between plasma lipoproteins. Taking into account the well-documented anti-inflammatory properties of HDL,[9, 10] upon LPS administration, reduced plasma CETP concentration results in rapidly increased HDL-C. Thus, presence of CETP may play a beneficial role in the acute neutralization of LPS, whereas down regulation of CETP expression immediately following the exposure increases the level of anti-inflammatory HDL. Intriguingly, as compared to wild-type mice naturally deficient of CETP, CETP transgenic mice have a lower plasma HDL-C level.[11, 24] It is thus tempting to speculate that the presence of CETP increases the flexibility of the organism to respond to inflammatory stimuli like LPS.

In fact, in APOE*3-Leiden.CETP mice we observed that 8 hours after LPS injection, significantly enhanced the HDL-C/nonHDL-C ratio, which follows the rapidly decreased plasma level and hepatic expression of CETP. At 48 hours after LPS injection, plasma CETP concentration and hepatic CETP gene expression were still lower compared to those of the control group, however, plasma HDL-C and TG were normalized, suggesting a transient effect on lipoprotein metabolism in response to LPS mediated CETP reduction. These data indicate that more processes involved in lipoprotein metabolism than CETP expression alone are affected by LPS exposure mice.[25]

Since HDL-C level is inversely correlated with CVD risk, and because of the crucial role in HDL metabolism, CETP has been considered as a promising target for treatment of dyslipidemia and CVD during the last decade. Indeed, inhibiting CETP activity by small molecular inhibitors, such as torcetrapib,[2] dalcetrapib,[3] anacetrapib[26] and evacetrapib,[27] all

have been shown to increase HDL-C. In addition, torcetrapib, anacetrapib and evacetrapib, but not dalcetrapib, have been shown to reduce LDL-C. As opposed to the convincing favorable effects on lipoprotein profile, the clinical outcomes of the CETP inhibitors on CVD events are very disappointing. Clinical development of dalcetrapib and evacetrapib was stopped because of lack of clear clinical benefit, while torcetrapib was stopped in a phase III clinical trial due to severe off-target effects and pro-inflammatory lesions.[28] In the present study, we observed a strong association between hepatic expression of CETP and markers of Kupffer cell maturation after LPS administration. This association is in full agreement with the finding from our previous study (**chapter 3**) that the reappearance of CETP expression is paralleled with expression of markers of Kupffer cell maturation after Kupffer cell depletion by liposomal clodronate. Collectively, these data suggest that CETP genes and genes related to Kupffer cell maturation and activation may be (partially) regulated by the same regulatory pathways.

In conclusion, our findings show that LPS reduces plasma concentration and hepatic expression of CETP by activation of Kupffer cells, without affecting total hepatic macrophage content. This rapid response to decrease plasma CETP concentration and as a consequence to raise HDL-C may play a role in host defense via the anti-inflammatory effects of HDL-C. Furthermore, the strong association between the expression in Kupffer cells of CETP and markers of Kupffer cell maturation/activation is important for future evaluation of the effects of CETP inhibition on inflammatory status of the liver.

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